# THE EFFECT OF NUTRIENT MEDIA UPON HEAD FREQUENCY IN REGENERATING PLANARIA [1]

S. J. COWARD, R. A. FLICKINGER AND E. GAREN [2]

*Department of Zoology, University of California, Davis, California*

The term "head frequency," as used by Child (1941), refers to the completeness and rate of differentiation of head structures in regenerating planaria. In most species of planarians the regeneration of head structures is less complete and occurs more slowly at successively more posterior levels. Heads regenerated at progressively posterior levels are smaller, until no head regeneration at all occurs at the extreme caudal end of the worm. There is also an anterior-posterior gradient of rate of regeneration of head structures, and this can be visualized most easily by comparing the time required after cutting the worms for the formation of eyespots at different levels of the worm.

Quantitative anterior-posterior gradients of incorporation of $C^{14}$-labeled $CO_2$ and glycine into the protein fraction of planaria (*Dugesia tigrina* and *D. dorotocephala*) have been demonstrated (Flickinger, 1959). These gradients can be abolished by treating the worms with chloramphenicol or colcemide (deacetylmethylcolchicine) for one or two days. Furthermore, the polarity of regenerating worms can be reversed by embedding the cut pieces in agar slabs and then immersing the prospective anterior end of the worm into a solution of chloramphenicol or colcemide (Flickinger, 1959; Flickinger and Coward, 1962). Presumably the gradient has been altered by this treatment, inhibiting the prospective head end of the cut piece, thus allowing cephalic differentiation at the prospective caudal end.

These experiments illustrate that reversal of the normal gradient of protein synthesis reverses the polarity of the regenerating worm, but such experimental control of polarity is of a negative nature. On the other hand, Brønsted and Brønsted (1953) have shown that addition of yeast sodium ribonucleate to starved decapitated planaria can accelerate the time of eyespot formation, which suggests that certain metabolites playing a part in protein synthesis are not present at optimal levels in starved planaria. It is known that the head frequency (*i.e.*, extent and rate of head formation) is greatest in anterior regions, most easily evidenced by the fact that eyespots form later in the posterior regions. The results of Brønsted and Brønsted (1953) suggested that treatment of sections from different body levels with sodium ribonucleate, amino acids, or other substances that might promote protein synthesis, would alter the head frequency gradient.

## Materials and Methods

The planaria, *Dugesia dorotocephala*, were obtained from a commercial supplier and were starved for various periods of time before being used. Before

[1] This investigation was supported by grants from the National Science Foundation (G-19963) and a University of California Cancer Research Grant.

[2] Mr. Garen was supported by a National Science Foundation summer training program in biology for high school students.

cutting, the worms were washed several times with boiled tap water, exposed to bactericidal ultraviolet light for five minutes and then cut with cataract knives. The heads and tails were removed and the rest of the worm was cut into three or six pieces of equal size (Fig. 1). In tabulating the results the pieces are numbered in an anterior-posterior direction. In some experiments with the worms cut into six pieces, the middle pieces (3 and 4) containing the pharynx were not used. In those worms cut into three parts, the pieces corresponding to pieces 1 + 2, 3 + 4 and 5 + 6 of the worms divided into six parts. The horse serum, chick embryo extract, vitamin and amino acid mixtures (essential and non-essential amino acids

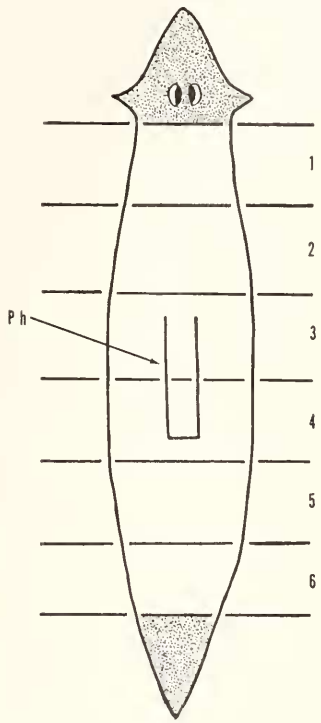


FIGURE 1. Diagram showing the regions used for regeneration experiments after removal of the head and tail. The pieces (3, 4) containing the pharynx (Ph) were not used in some of the experiments.

of Eagle's basal medium) were obtained commercially; the horse serum and chick embryo extract were shipped in the frozen state. A penicillin-streptomycin mixture (1%) was present in each of the media, including the boiled tap water.

In the first type of experiment the compounds to be tested were dissolved together in a 1/10 dilution of Niu-Twitty saline (Niu and Twitty, 1953) to provide a complex nutrient medium. In the other experiments these fractions were dissolved in Seitz-filtered tap water and tested individually. In two series of experiments with yeast sodium ribonucleate, cut sections were exposed only to sodium RNA for one day after cutting, subsequently being transferred to boiled tap water.

In all other experiments the cut worms were maintained continually in the test medium or boiled tap water in small Stender dishes. The pH's of the various media were 7.2 to 8.0. The regenerating worms were maintained at 20° C. and transferred to fresh media every second day.

## Results

*Experiments with complex nutrient medium; continual exposure*

The heads and tails of 30 worms that had been starved for 5 days were cut away and the remaining midsection cut into three pieces of equal size. The cut sections from 15 of these worms were cultured in boiled tap water containing 1% penicillin-streptomycin; the cut pieces of 15 other worms were cultured in a 1/10 dilution of Niu-Twitty medium containing 5% horse serum, 1% chick embryo extract, 1% vitamin mixture, 0.5% concentration of both the essential and non-

Table I

*Regeneration of eyespots in nutrient medium versus tap water; worms starved 10 days*

| Medium | Level of section* | Number of sections | Number with eyespots | | | |
|---|---|---|---|---|---|---|
| | | | 5 | 6 | 7 | 8 |
| | | | Days after cutting | | | |
| A. Tap water | 1 + 2 | 15 | 10 | 13 | 15 | 15 |
| B. Nutrient medium** | 1 + 2 | 14 | 11 | 14 | 14 | 14 |
| A. Tap water | 3 + 4 | 13 | 1 | 2 | 5 | 6 |
| B. Nutrient medium | 3 + 4 | 15 | 9 | 13 | 14 | 14 |
| A. Tap water | 5 + 6 | 13 | 1 | 6 | 7 | 7 |
| B. Nutrient medium | 5 + 6 | 15 | 7 | 12 | 14 | 14 |

* The numbers refer to the various levels shown in Figure 1.

** The nutrient medium was composed of 5% horse serum, 0.5% amino acids, 1% chick embryo extract, 1% vitamin mixture and 0.2% glucose in sterile tap water.

essential amino acid mixtures of Eagle's medium, 1% glutamine, 0.2% glucose and 1% penicillin-streptomycin under a 95% air–5% $CO_2$ atmosphere. Observation of these cut worms revealed that the head frequency was higher in the anterior (number 1) as compared to the middle (number 2) and posterior (number 3) sections. However, the nutrient medium had no significant effect in accelerating the time of eyespot appearance or the number of eyespots differentiating at any of the three levels.

This experiment was then repeated with worms that had been starved for 10 days instead of 5 days. In this experiment both the extent and time of appearance of eyespots in the middle and posterior pieces (levels 2 and 3) were stimulated in those regenerates maintained in the nutrient medium, as compared to the controls kept in tap water, but there was no effect upon the anterior pieces (level 1)

(Table II). Six days after cutting, 13 of the 15 cut midsections in the nutrient medium had formed eyespots, while only 2 of 13 in tap water had formed eyespots; 12 of 15 of the posterior pieces in nutrient had formed eyespots, *versus* 6 of 13 pieces in tap water. Thus, the frequency of eyespot formation, as well as the rate, is influenced by the nutrient medium. Eight days after cutting of the most posterior sections (level 3), 14 of 15 pieces in the nutrient medium possessed eyespots; at this same time eyespots were present in only 7 of 13 pieces that regenerated in tap water. Subsequent observation for another week revealed no further differentiation of eyespots in these worms.

*Experiments with sodium ribonucleate, one-day exposures*

In this series of experiments 45 planaria were starved for 14 days, the heads and tails extirpated and the remainders of these worms were cut into six pieces of equal size (Fig. 1). Fifteen sections from each level were used in each of three test solutions: (1) 0.01% sodium ribonucleate, (2) 0.005% sodium ribonucleate, and (3) boiled tap water. The worms were exposed to sodium RNA solutions for

TABLE II

*Regeneration of eyespots after a one-day exposure to yeast sodium ribonucleate; worms starved for 14 days*

| Medium | Level of section | Total number of sections | Number with eyespots | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4 | 5 | 6 | 7 | 8 | 9 |
| | | | Days after cutting | | | | | |
| A. Sodium RNA (0.01%) | 1 | 12 | 5 | 7 | 10 | 11 | 11 | 11 |
| B. Sodium RNA (0.005%) | 1 | 14 | 8 | 11 | 13 | 13 | 13 | 13 |
| C. Tap water | 1 | 14 | 4 | 6 | 11 | 13 | 13 | 13 |
| A. | 2 | 14 | — | 1 | 1 | 2 | 3 | 3 |
| B. | 2 | 14 | — | 2 | 2 | 2 | 3 | 3 |
| C. | 2 | 14 | — | 1 | 2 | 5 | 6 | 6 |
| A. | 3 | 12 | — | 1 | 1 | 1 | 1 | 1 |
| B. | 3 | 14 | — | 2 | 2 | 2 | 3 | 3 |
| C. | 3 | 13 | — | — | 1 | 1 | 1 | 1 |
| A. | 4 | 15 | — | — | — | — | 1 | 1 |
| B. | 4 | 15 | — | 1 | 1 | 1 | 1 | 2 |
| C. | 4 | 14 | — | — | — | — | — | — |
| A. | 5 | 13 | — | 2 | 3 | 3 | 3 | 4 |
| B. | 5 | 14 | — | 1 | 1 | 1 | 1 | 1 |
| C. | 5 | 14 | — | — | 1 | 1 | 1 | 1 |
| A. | 6 | 15 | — | 2 | 8 | 12 | 12 | 12 |
| B. | 6 | 13 | — | 6 | 9 | 11 | 11 | 12 |
| C. | 6 | 14 | — | 2 | 5 | 6 | 6 | 6 |

TABLE III

*Regeneration of eyespots after a one-day exposure to yeast sodium ribonucleate; worms starved for five days*

| Medium | Level of worm | Total number of sectioned pieces | Number with eyespots | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | Days after cutting | | | | | | |
| A. Sodium RNA (0.01%) | 1 | 14 | 1 | 4 | 5 | 6 | 6 | 7 | 9 |
| B. Sodium RNA (0.005%) | 1 | 15 | 1 | 3 | 6 | 8 | 10 | 11 | 11 |
| C. Tap water | 1 | 15 | — | 3 | 6 | 8 | 9 | 10 | 11 |
| A. | 2 | 14 | — | — | — | 1 | 2 | 2 | 2 |
| B. | 2 | 14 | — | — | — | 2 | 3 | 4 | 4 |
| C. | 2 | 15 | — | — | — | — | — | 1 | 2 |
| A. | 5 | 15 | — | 2 | 2 | 2 | 2 | 2 | 2 |
| B. | 5 | 15 | — | — | — | — | — | — | — |
| C. | 5 | 15 | — | — | — | 1 | 1 | 1 | 1 |
| A. | 6 | 15 | — | 3 | 9 | 11 | 11 | 11 | 11 |
| B. | 6 | 15 | — | 7 | 13 | 13 | 13 | 13 | 13 |
| C. | 6 | 15 | — | 3 | 5 | 7 | 9 | 9 | 10 |

the 24-hour period after cutting, and then transferred to boiled tap water. Regeneration was followed up to 20 days after cutting, but differentiation was complete 9 days after cutting. The results are shown in Table II. In the most posterior sections of the worms (level 6), both concentrations of sodium RNA seemed to have stimulated a higher incidence of eyespot differentiation seven days after sectioning the worms.

This experiment was repeated in the same way with a group of worms that had been starved for five days, and stimulation of time of eyespot formation occurred at the posterior level. These results are presented in Table III. The results of cut sections 3 and 4 are omitted in the table because these regions regenerate poorly, presumably due to the mechanical interference of the pharynx. The effects of RNA in speeding the time of differentiation of the eyespots are evident at level 6, and this effect is demonstrated to a slight extent at level 2.

### Experiments with Horse Serum, Amino Acids, Chick Embryo Extract, Sodium Ribonucleate and Tap Water: Continual Exposure

From worms starved for 30 days, 10 cut sections from each of the four regions 1, 2, 5 and 6 (Fig. 1) were cultured in each of the following separate solutions: 5% horse serum, 0.5% essential plus 0.5% non-essential amino acids of Eagle's medium, 1% chick embryo extract, 0.005% yeast sodium ribonucleate and boiled tap water. These media were diluted to the test concentrations by addition of Seitz-filtered tap water which contained a 1% penicillin-streptomycin mixture. The results are given in Table IV. It is evident that the normal gradient of head fre-

TABLE IV

*Regeneration of eyespots upon continuous exposure to various nutrient solutions and tap water; worms starved for 30 days*

| Medium | Level of worm | Total number of sectioned pieces | Number with eyespots | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | | | Days after cutting | | | | | | | | |
| H.S.—Horse serum (5%) | 1 | 9 | 1 | 3 | 5 | 7 | 7 | 7 | 7 | 8 | 8 |
| A.A.—Eagle's essential and non-essential amino acids (0.5% each) | 1 | 7 | 1 | 2 | 4 | 4 | 5 | 5 | 5 | 5 | 6 |
| E.E.—Chick embryo extract (1%) | 1 | 7 | 1 | 3 | 5 | 7 | 7 | 7 | 7 | 7 | 7 |
| RNA—Yeast sodium ribonucleate (0.005%) | 1 | 7 | — | 3 | 6 | 7 | 7 | 7 | 7 | 7 | 7 |
| $H_2O$—Boiled tape water | | 8 | — | — | 2 | 3 | 3 | 5 | 5 | 6 | 7 |
| H.S. | 2 | 8 | — | — | 2 | 2 | 2 | 2 | 3 | 3 | 3 |
| A.A. | 2 | 8 | — | 1 | 2 | 3 | 5 | 5 | 6 | 6 | 6 |
| E.E. | 2 | 5 | — | — | 1 | 1 | 2 | 3 | 3 | 4 | 4 |
| RNA | 2 | 10 | — | 1 | 2 | 2 | 4 | 4 | 5 | 6 | 6 |
| $H_2O$ | 2 | 8 | — | — | 1 | 2 | 2 | 3 | 4 | 5 | 6 |
| H.S. | 5 | 8 | — | — | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| A.A. | 5 | 6 | — | 1 | 3 | 3 | 4 | 4 | 4 | 4 | 4 |
| E.E. | 5 | 10 | — | — | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| RNA | 5 | 9 | — | — | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| $H_2O$ | 5 | 10 | — | — | 1 | 1 | 3 | 3 | 3 | 3 | 4 |
| H.S. | 6 | 8 | 1 | 3 | 5 | 5 | 7 | 7 | 7 | 7 | 7 |
| A.A. | 6 | 8 | — | 2 | 4 | 4 | 6 | 6 | 6 | 6 | 6 |
| E.E. | 6 | 8 | — | 2 | 4 | 4 | 4 | 6 | 7 | 8 | 8 |
| RNA | 6 | 10 | — | 1 | 3 | 3 | 4 | 4 | 5 | 6 | 8 |
| $H_2O$ | 6 | 10 | — | — | 2 | 5 | 5 | 5 | 6 | 6 | 6 |

quency that is observed in worms regenerating in tap water is modified in sections 1 and 6 by each of the test solutions, as shown by acceleration of the time of eyespot differentiation. In these experiments the extent of eyespot regeneration did not seem to vary between the nutrient solutions and tap water controls at the various levels.

## Isotopic Experiments with Worms Exposed to Nutrient Medium

In order to test the assumption that the nutrient medium had stimulated protein synthesis, studies using labeled $CO_2$ were undertaken. Worms starved for three weeks were divided into two groups of 60. Group I was cut to provide three regenerating pieces, each with two wound surfaces, per intact worm (1 + 2, 3 + 4, 5 + 6; Fig. 1). Group II had only the heads and tails removed to provide one regenerating piece, each with two wound surfaces, per intact worm. Each group was

divided in half, 30 pieces of each kind being cultured in nutrient medium and 30 in Seitz-filtered tap water. The nutrient medium was the same as previously used, but horse serum was omitted and exposure lasted four days. At the end of this period the regenerating pieces were exhaustively washed over a four-hour period and then incubated separately, each with 150 $\mu$c. $C^{14}O_2$, for three hours, according to the method of Flickinger (1959). The incubation was terminated by the addition of cold 10% TCA. The regenerating pieces of Group II were cut in three pieces, pre-pharyngeal, pharyngeal and post-pharyngeal, which corresponded to the three levels of Group I. Following homogenization and washing with 10% TCA, the residue was treated with hot 5% TCA to hydrolyze nucleic acids, and then lipids were extracted. The residue, designated as the protein fraction, was dissolved in 0.5 ml. 0.2 N $NH_4OH$, plated on tared planchets, weighed, and counted in a gas-flow counter. Self-absorption was not a significant source of error. The results of this experiment (Table V) clearly demonstrate that stimu-

TABLE V

*Effect of nutrient medium on stimulation of $C^{14}O_2$ incorporation into protein of regenerating worms; worms starved three weeks*

| Medium | Level of section* | Group I | | Group II | |
|---|---|---|---|---|---|
| | | CPM/MG Protein | % Stimulation | CPM/MG Protein | % Stimulation |
| Tap water | 1 + 2 | 8,928 | — | 9,672 | — |
| | 3 + 4 | 7,896 | — | 9,139 | — |
| | 5 + 6 | 6,169 | — | 6,502 | — |
| Nutrient medium** | 1 + 2 | 2,776 | −322 | 15,605 | +161 |
| | 3 + 4 | 9,947 | +126 | 6,976 | −131 |
| | 5 + 6 | 11,545 | +187 | 19,770 | +304 |

* See Figure 1.

** The nutrient medium was the same as in Table I except for the omission of horse serum.

lation of protein synthesis discernible by $C^{14}O_2$ incorporation is greatest in the posterior levels. It is noteworthy that the axial gradient of incorporation (Flickinger, 1959) is preserved in the tap water controls, but markedly altered in those from nutrient medium.

## DISCUSSION

The results of this investigation clearly support previous work (Child, 1941) and demonstrate that an anterior-posterior gradient of head frequency exists for the time of appearance and completeness of development of eyespots in pieces of regenerating planaria. Furthermore, the data indicate that if sodium ribonucleate, horse serum, amino acids, or chick embryo extract are added to the medium, the number of eyespots forming and the rate of their appearance are usually increased, particularly at the posterior levels of the worm. This effect is more striking in worms that have been starved for longer periods, but even in starved worms the extent of eyespot formation is sometimes not affected, although

the rate of eyespot regeneration is stimulated. Regeneration is usually quite poor in pieces in which the pharynx occupies the majority of the piece; thus such regions were not utilized in some of the experiments.

Some attempts were made to reverse the polarity of regenerating pieces by immersing the prospective posterior end of the worms in 0.005% sodium ribonucleate in the agar-slab type of experiment (Flickinger, 1959), but these experiments were unsuccessful. The compounds used in this study, which we have designated as nutrients, can accelerate differentiation of cephalic structures, an effect which is more pronounced at posterior levels. Despite the fact that these nutrients cannot be demonstrated to affect polarity in the agar-slab experiments, as chloramphenicol does, they do stimulate the rate and extent of cephalic regeneration, suggesting that their availability influences the rate of protein synthesis. Previous mention has been made of the evidence relating a gradient of protein synthesis to the biological polarity in the regenerating planarian (Flickinger, 1959; Flickinger and Coward, 1962).

The results of our earlier experiments show that this stimulation is greater at posterior levels of the worms where isotopic data show a lower rate of incorporation of $CO_2$ and glycine into protein (Flickinger, 1959). The present isotopic data demonstrate that nutrients stimulate protein synthesis asymmetrically in respect to the normal axial gradient. This is in harmony with the observed biological effect—the increase of head frequency at posterior levels. The fact that substrates and cofactors necessary for protein synthesis can stimulate the rate of eyespot formation in cut posterior regions of the worms more than at the anterior end suggests that these materials are not present at optimal levels in the posterior parts of the worms. This contention is in agreement with the hypothesis that head formation occurs at the cut end of the worm with the highest level of activity of the protein-synthesizing mechanisms. Furthermore, the inhibitory role of the head in preventing posterior cephalic differentiation may be due to its capacity to drain substrates and cofactors from the more posterior levels.

## Summary

1. Planaria, *Dugesia dorotocephala,* were starved for periods of 5–30 days, the heads and tails removed and the remainders of the worms were cut into three, four or six pieces. These sections were allowed to regenerate in tap water or in solutions containing yeast sodium ribonucleate (0.005–0.01%), horse serum (5%), amino acids (1%), and chick embryo extract (1%). The media were changed every second day. The cultures were observed daily and the time of eyespot formation was recorded.

2. All of the nutrient solutions tested were effective in promoting the rate of regeneration of eyespots, particularly in the posterior pieces in which eyespots appeared 1–2 days sooner than in the tap water controls. The effect of the test solutions upon the extent of eyespot regeneration is less consistent, but some stimulation of differentiation at the posterior levels was observed.

3. Exposure to yeast sodium ribonucleate for only a day after cutting the worms also stimulated head frequency posteriorly, but this is not as effective as continual exposure.

4. The biological results were confirmed by $C^{14}O_2$ incorporation studies which demonstrated increased synthetic activity in posterior levels of the worms that had been exposed to the nutrient medium.

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